



Multiplex Touchdown PCR for Rapid Typing of the Opportunistic Pathogen *Propionibacterium acnes*

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1 **Title:**

2 Multiplex Touchdown PCR for Rapid Typing of the Opportunistic Pathogen
3 *Propionibacterium acnes*

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5 **Running head:**

6 Multiplex PCR typing of *P. acnes*
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21 ABSTRACT

22 The opportunistic human pathogen *Propionibacterium acnes* is comprised of a number of
23 distinct phylogroups, designated types IA₁, IA₂, IB, IC, II and III, that vary in their
24 production of putative virulence factors, inflammatory potential, as well as biochemical,
25 aggregative and morphological characteristics. Although Multilocus Sequence Typing
26 (MLST) currently represents the gold standard for unambiguous phylogroup classification,
27 and individual strain identification, it is a labour and time-consuming technique. As a
28 consequence, we have developed a multiplex touchdown PCR assay that will, in a single
29 reaction, confirm species identity and phylogeny of an isolate based on its pattern of reaction
30 with six primer sets that target the 16S rRNA (all isolates), ATPase (type IA₁, IA₂, IC), *sodA*
31 (type IA₂, IB), *atpD* (type II) and *recA* (type III) housekeeping genes, as well as a Fic family
32 toxin gene (type IC). When applied to 312 *P. acnes* isolates previously characterised by
33 MLST, and representing type IA₁ (n=145), IA₂ (n=20), IB (n=65), IC (n=7), II (n=45) and III
34 (n=30), the multiplex displayed 100% sensitivity and 100% specificity for the detection of
35 isolates within each targeted phylogroup. No cross-reactivity with isolates from other
36 bacterial species was observed. The multiplex assay will provide researchers with a rapid,
37 high-throughput and technically undemanding typing method for epidemiological and
38 phylogenetic investigations. It will facilitate studies investigating the association of lineages
39 with various infections and clinical conditions, as well as a pre-screening tool to maximise
40 the number of genetically diverse isolates selected for downstream, higher resolution
41 sequence-based analyses.

42 INTRODUCTION

43 *Propionibacterium acnes* is an anaerobic-to-aerotolerant Gram-positive bacterium which
44 exists in nature as a human commensal and opportunistic pathogen. It is a major component
45 of the human skin microbiota, but can also be recovered from the oral cavity, gastrointestinal
46 and genitourinary tracts (1). Although *P. acnes* is the main cause of opportunistic human
47 infections within the 'cutaneous' group of propionibacteria, and is well known for its
48 association with the inflammatory skin condition acne vulgaris (2, 3), its role in other human
49 infections and clinical conditions is likely to have been significantly underestimated (4-6).
50 Despite this, we now see a growing recognition that the bacterium is an important cause of
51 human disease, especially in relation to indwelling medical device-related infections (7-12),
52 and may also play a role in chronic conditions that cause significant morbidity and mortality,
53 including low back pain associated with modic type I changes (13), sarcoidosis (14, 15) and
54 prostate cancer (16, 17).

55 Within the last ten years, phylogenetic studies based on single and multilocus gene
56 sequencing (18-21), as well as whole genome analyses of isolates from the Human
57 Microbiome Project (HMP) and other studies (22-28), have provided valuable insights into
58 the genetic population structure of *P. acnes*, particularly in the context of health and disease.
59 The bacterium has an overall clonal structure and isolates can be classified into a number of
60 statistically significant clades or phylogroups, designated types IA₁, IA₂, IB, IC, II and III,
61 which appear to display differences in their association with specific types of infections (20-
62 21), and also vary in their production of putative virulence determinants (19, 20, 29-32),
63 inflammatory potential (33-36), antibiotic resistance (21, 37), aggregative properties (16) and
64 morphological characteristics (19). In particular, a number of independent epidemiological
65 studies have shown a strong association between clonal complexes from the type IA₁
66 phylogroup and moderate-to-severe acne, while lineages from all other divisions appear more

67 frequently isolated from medical device and soft tissue infections, or associated with health as
68 true commensals (20, 21, 31, 38). Despite these associations, much uncertainty still exists
69 regarding their exact clinical relevance, particularly in the context of acne where skin
70 sampling methods may not be optimal or appropriate (39), as well as the wider issue of
71 whether isolates recovered from different clinical samples are truly representative of infection
72 in all contexts, or are simply skin contaminants/ passive bystanders within a sample; such
73 issues are common when dealing with an opportunistic pathogen that is also part of the
74 normal microbiota, and untangling clinically relevant isolates from background contaminants
75 can be a challenge. Future studies aimed at addressing such issues will undoubtedly provide a
76 more solid platform on which we can make definite conclusions regarding the association of
77 specific *P. acnes* phylogroups with human disease.

78 To date, a number of phenotypic and/ or molecular approaches have been investigated
79 as methods for phylogroup identification, ranging from very simple biochemical tests based
80 on haemolysis (19) or fermentation profiles (40), to matrix assisted laser
81 desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (41), monoclonal
82 antibody (mAb) typing (18) and DNA-based analysis; the latter includes ribotyping (23),
83 DiversiLab analysis (42), direct PCR assays (43) and protein-encoding gene sequencing (18,
84 20, 21, 44). Unfortunately, many of these approaches suffer from specificity or sensitivity
85 limitations, which constrain their diagnostic value. For example, previously described direct
86 PCR assays do not differentiate IA₁ from type IA₂ or IC, or target type III strains (43); they
87 may also give ambiguous results (44). Single locus nucleotide sequencing, which to date has
88 been primarily based on the *recA* housekeeping and *tly* methyltransferase/ haemolysin genes,
89 is robust for identification of types I, II and III, but displays reduced specificity for
90 differentiation of type IB strains from type IA₂ and some strains within the type IA₁ clade as
91 they contain identical alleles due to horizontal gene transfer (HGT) (20, 21, 23, 38). More

92 recently, mAb typing with antibodies targeting types IA, IC and II, combined with *recA*
93 sequence analysis has obviated this problem, facilitating accurate differentiation of type IB
94 from all type IA strains (45). MALDI-TOF MS has also been described as a valuable and
95 powerful approach for rapid and high throughput phylogroup identification, but currently will
96 not differentiate type IA₁ from IA₂ (41); furthermore, the technology is not available within
97 all laboratories. At present, Multilocus Sequence Typing (MLST) of *P. acnes* (20, 21) still
98 represents the clear gold standard for unambiguous phylogroup identification, as well as
99 individual strain resolution (Table S1), and offers significant advantages over comparable
100 high resolution gel-based typing methodologies, including Random Amplification of
101 Polymorphic DNA (RAPD) (46) and Pulse Field Gel Electrophoresis (PFGE) (47, 48) which
102 have also been applied to this bacterium. The MLST method is, however, technically
103 demanding, time consuming and expensive, especially when analysing multiple isolates.
104 Consequently, and as a result of the growing interest surrounding the role of this microbe in
105 disease, there is a need for a rapid, less labour intensive and inexpensive method for typing
106 and stratification of *P. acnes* isolates, thus facilitating future molecular epidemiological and
107 phylogenetic studies. Against this background, we now describe the development and
108 validation of a multiplex touchdown PCR assay that can be used for quick, high-throughput
109 and accurate molecular confirmation of *P. acnes* isolates combined with parallel disclosure of
110 their phylogeny. This assay should prove valuable for researchers and, along with MLST,
111 form part of a 'molecular typing toolbox' that can be used for the analysis of *P. acnes*
112 isolates.

113

114 MATERIALS AND METHODS

115 **Bacterial strains and growth.** A total of 312 *P. acnes* isolates were used to validate the
116 multiplex PCR (145 type IA₁; 20 type IA₂, 65 type IB, 7 type IC, 45 type II and 30 type III).
117 These isolates were previously recovered from a wide range of clinical sources and healthy
118 skin, and their phylogroup status determined using an MLST scheme based on eight genes
119 (38). A representative sample of isolates from each group were also analysed by mAb typing
120 as previously described (18), and their reactivity was consistent with their phylogroup
121 designations based on MLST. Genomic DNA from a panel of 49 isolates representing 34
122 medically relevant bacterial species, and including other human *Propionibacterium* species,
123 was also used in the assessment of multiplex specificity (Table S2). All bacterial strains were
124 maintained at -80 °C in brain heart infusion (BHI) broth, containing 12% (v/v) glycerol.
125 Anaerobic isolates were cultured in BHI and on anaerobic horse blood agar (ABA) plates
126 (Oxoid Ltd, Hampshire, UK) at 37 °C in an anaerobic cabinet (Mark 3; Don Whitley
127 Scientific) under an atmosphere of 10% H₂, 10% CO₂, 80% N₂. Aerobic bacteria were
128 cultured on horse blood agar at 37 °C.

129
130 **Development of phylogroup-specific primers.** Housekeeping gene sequences representing
131 *aroE* (424bp), *atpD* (453bp), *gmk* (400bp), *guaA* (493bp), *lepA* (452bp) and *sodA* (450bp)
132 were retrieved from the *P. acnes* MLST database (<http://pubmlst.org/pacnes/>). Sequences for
133 each gene were then aligned using MEGA v5.1 software and inspected for phylogroup-
134 specific polymorphisms. Phylogroup-specific genomic regions were also investigated using
135 the progressiveMauve algorithm (v2.3.1) and the Artemis Comparison Tool (ACT;
136 <http://www.sanger.ac.uk/Software/ACT/>) using whole genome sequences (WGS) currently
137 available as part of the HMP, as well as other sequencing projects
138 (<http://www.ncbi.nlm.nih.gov/genome/genomes/1140>) (Table S1). Based on these analyses,

139 phylogroup-specific primer sets were developed as listed in Table 1.

140

141 **Multiplex PCR analysis.** Bacterial genomic DNA was prepared using an AquaGenomic™
142 kit (Multi Target Pharmaceuticals). PCR amplification was carried out using a MultiGene
143 thermocycler (Labnet International Inc, UK). Samples contained 1 x PCR buffer, 200 µM of
144 each deoxynucleoside triphosphate (Invitrogen Life Technologies, UK), six primer sets
145 targeting each phylogroup at concentrations described in Table 1, 1.5 mM MgCl₂, 1x
146 *RediLoad*™ (Invitrogen Life Technologies), 1.25 U *Taq* DNA polymerase (Invitrogen Life
147 Technologies) and 1µl of pure genomic DNA preparation in a total volume of 10 µl. Samples
148 were initially heated at 94°C for 1 min, followed by 14 cycles consisting of 94°C for 30 s,
149 66°C (decreasing incrementally 0.3°C per cycle) for 30 s, and 72°C for 1 min, followed by 11
150 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 1 min, culminating with a final cycle at
151 72°C for 10 min. A negative control (PCR water) and six positive control samples
152 representing all phylogroups were included in all experiments. PCR products were analysed
153 by electrophoresis on 1.5% (w/v) agarose gels containing 1x Tris-acetate-EDTA buffer.
154 Molecular size markers were run in parallel on all gels. Resolved DNA products were stained
155 with 1x GelRed™ Nucleic Acid Gel stain (Cambridge Biosciences, UK).

156

157 **Nucleotide sequencing.** Sequencing reactions were performed using BigDye® reaction
158 terminator cycle sequencing kits (version 1.1) (Life Technologies, UK) according to the
159 manufacturer's instructions. Samples were then analysed on an ABI PRISM 3100 genetic
160 analyser capillary electrophoresis system (Life Technologies).

161

162 **Split decomposition analysis.** Split decomposition analysis was performed using SplitsTree4
163 version 4.13.1 (49)

164 **RESULTS**

165 **Primer design.** Polymorphisms in multiple aligned sequences of the *sodA* gene specific for
166 types IA₂ and IB, and *atpD* and *recA* genes specific to types II and III, respectively, were
167 identified as candidate regions for primer development (Table 1). Primers were also
168 developed against the ATP-binding component (ATPase; GenBank accession no.
169 ABB20821.1) of a previously described ABC-type peptide uptake operon (DQ208967)
170 present in the closely related type IA₁, IA₂ and IC groups, but absent in type IB, II and III
171 strains (Table 1) (43). This operon also includes genes encoding permeases (ABB20819.1;
172 ABB20820.1) and a solute binding protein (ABB20823.1), alongside genes for a glycoside
173 hydrolase (ABB20818.1) and chitinase (ABB20824). For type IC strains, we developed a
174 primer set targeting a Fic family-toxin gene located on an approximately 7.3 kb genomic
175 fragment present in the draft genome sequences of the type IC strains PRP-38
176 (TICEST70_07737) and HL097PA1 (HMPREF9344_02057), but not other phylogroups
177 (Table 1). This genomic fragment also contained restriction enzyme-associated genes and a
178 gene encoding a DEAD/DEAH box helicase (HMPREF9344_02061). Our previously
179 described *P. acnes*-specific 16S rDNA-based primers were also included in the assay to
180 confirm species identity (Table 1) (11, 50). Primer sets incorporated phylogroup-specific
181 mismatches at the 3' end, and elsewhere in the sequence when available, and were designed
182 to have identical annealing temperatures where possible, and to generate amplicons with
183 characteristic size differences that would facilitate easy visual identification on a gel after
184 multiplexing.

185
186 **Multiplex PCR development and validation.** Each individual phylogroup-specific primer
187 set was initially examined against a small panel of strains (n=40) representing types IA₁, IA₂,
188 IB, IC, II and III. Amplicons of predicted size were correctly generated from the targeted

189 phylogroup, and no products were unexpectedly observed in divisions outside those targeted
190 by the primers (data not shown). The identity of each PCR product was confirmed by direct
191 nucleotide sequence analysis (data not shown). Each primer set was then combined into a
192 single multiplex touchdown PCR reaction, which was optimised for final primer and MgCl_2
193 concentrations, as well as amplification cycles, as outlined in the methods section. As the
194 *sodA* primers PAMp-3/ PAMp-4 had a lower annealing temperature (62°C) than all the other
195 primer sets (66°C), a touchdown PCR approach was adopted to ensure satisfactory, highly
196 specific amplification of all gene targets within the assay. Using this approach, it proved
197 possible to reliably determine the phylogeny of an isolate based on the combination of
198 different phylogroup-specific amplification products as illustrated in Fig 1.

199 To assess the sensitivity and specificity of the multiplex assay, especially in relation
200 to primers targeting genomic regions that were presumptively present/absent between
201 phylogenetic divisions based on *in silico* analysis of WGS data, we screened a large panel of
202 312 *P. acnes* isolates previously characterised by MLST, and representing a total of 97
203 unique sequence types (ST) covering all the phylogroups. Based on this current sample
204 cohort, the multiplex PCR displayed 100% specificity and 100% sensitivity for the detection
205 of isolates within each targeted phylogroup (Table 2); no cross-reactivity with isolates from a
206 wide range of other medically relevant bacterial species was observed, including other
207 cutaneous *Propionibacterium* and *Staphylococcus* species (Table 2 and Table S2).

208

209 **DISCUSSION:**

210 Since the 16S rRNA gene of *P. acnes* has very high intraspecific sequence identity (18, 19), it
211 afforded little opportunity for the design of phylogroup-specific primers on which we could
212 base our multiplex assay. As an alternative, we examined various protein-encoding
213 housekeeping loci, and interrogated available whole genome sequences representing all

214 known phylogroups, for unique genetic regions that could act as platforms for assay
215 development. By adopting this approach, we were able to design primers based on *atpD*
216 (PAMp-7/ PAMp-8) and *recA* (PAMp-9/ PAMp-10) housekeeping loci that specifically
217 identified phylogroup II and III strains, respectively. Type IC strains were identified by their
218 reaction with the primers PAMp-5/ PAMp-6 that targeted a Fic family toxin gene present on
219 a genomic region only found in type IC strains; such toxins form part of toxin-antitoxin (TA)
220 systems which are believed to be important in bacterial persistence in response to specific
221 environmental stresses, as well as pathogenicity (51). While types IA₁, IA₂ and IC all reacted
222 with primers targeting an ATPase gene (PAMp-1/ PAMp-2) that was part of an ABC-type
223 peptide uptake operon, differentiation of type IA₂ and IC strains from IA₁, and each other,
224 was achieved due to their separate reaction with the primers PAMp-3/ PAMp-4 and PAMp-5/
225 PAMp-6, respectively. The restriction of an ABC-type peptide uptake operon containing
226 chitinase to type IA and IC divisions, but not other phylogroups, is of particular interest and
227 may be potentially advantageous for the cleavage of chitin from the cell wall of the fungus
228 *Malassezia* and/or *Demodex* mites, which also colonise human skin (20). Since type IA₂
229 isolates contain alleles of the *sodA* locus that are identical (allele 4), or very closely related
230 (allele 5), to those present in all type IB strains (21, 38), both phylogenetic groups displayed
231 reaction with the *sodA* primer pair PAMp-3/ PAMp-4. Their identity was, however, easily
232 determined based on differential reaction with the ATPase primers PAMp-1/PAMp-2; type
233 IB isolates show no product with the latter primer set. Interestingly, as the type IA₂ clade
234 shares *recA* and *tly* alleles with type IB isolates it provides evidence for HGT of large
235 genomic fragments in the natural history of the bacterium (20, 21, 38).

236 By combining these different primer sets, along with the 16S rDNA primers PArA-1
237 and PArA-2, into a single multiplex assay, we have been able to provide researchers with a
238 robust method for rapid, high-throughput molecular identification of presumptive *P. acnes*

239 isolates, combined with valuable phylogenetic typing information. This assay should prove a
240 useful tool for epidemiological studies, as well as stratification of isolates for various
241 downstream analyses. It offers enhanced discrimination and specificity over comparable
242 molecular (PCR, single gene sequencing) and mAb typing approaches which have been
243 previously described in the literature, and is also less time consuming, especially over
244 methods that require multiple, separate analyses on each isolate. The multiplex PCR will
245 facilitate future retrospective and prospective studies aimed at investigating the association of
246 specific phylogenetic lineages with different human infections, clinical conditions and
247 antibiotic resistance, and will now also provide a technically undemanding way to rapidly
248 map multiple isolates from the same clinical sample so that the presence and pattern of mixed
249 population types can be determined, especially at different body sites/ niches. The method
250 should also provide a useful complement to the more detailed, and technically complicated,
251 study of *P. acnes* populations in complex microbiotas based on metagenomic analysis (23).
252 Although in this study we utilized purified genomic DNA as our template for multiplex PCR,
253 future optimization of the method for direct analysis of bacterial colonies (colony PCR)
254 would further enhance the rapid nature of the assay. Furthermore, of the *P. acnes* isolates
255 tested, numbers representing types IA₂ and IC were lower than those from other phylogroups,
256 especially types IC which are infrequently recovered. As a consequence, further analysis of
257 additional isolates from these clusters will be important to confirm the multiplex PCR
258 specificity and sensitivity results for these types.

259 To date, our understanding of *P. acnes* population structure within and between
260 different body habitats of individuals is poor. These sites not only include various areas of,
261 and regions within, the skin, but also the oral cavity and genitourinary tract. Such data may
262 prove especially valuable in our attempts to better understand the potential origin of different
263 lineages associated with clinical samples, particularly in relation to blood culture, and

264 whether the pattern of isolates recovered from primary surgical samples matches those on the
265 overlying/ surrounding skin, thus indicating potential contamination. In the surgical setting,
266 current methods used for pre-operative skin antisepsis do not always prevent microbial
267 contamination of surgical wounds with viable bacteria (52, 53). Although acute surgical site
268 infections may not ensue due to effective i.v. prophylactic antibiotic administration, these
269 bacteria may still cause downstream chronic biofilm-associated implant infection. It seems
270 reasonable to assume that contamination from the skin would result in a mixture of different
271 phylogenetic groups within a sample, while significant counts of a monotype may be more
272 indicative of infection. Under such circumstances, the multiplex could prove a valuable and
273 simple molecular screening tool to highlight such a scenario, and thus aid the diagnosis of
274 biofilm-associated implant infections and bacteremia etc within a clinical setting (54).
275 Furthermore, the detection of phylogroups with a potentially greater propensity to cause
276 infection within a clinical sample, such as IA₁, may also be more indicative of infection when
277 compared to phylogroups, such as types II and III, believed to be associated with a more
278 commensal existence (21).

279 To date, two MLST schemes based on eight (MLST₈) and nine (MLST₉) different
280 protein-encoding genes have been described for *P. acnes* (20, 21); the methods are essentially
281 concordant in respect to the clustering of strains into different clonal complexes (CCs),
282 although more subtle differences in the resolution of particular lineages within these CCs
283 exist (21, 38). While MLST provides high resolution typing of *P. acnes*, and generates not
284 only phylogroup information, but also ST data that is highly amenable to phylogenetic and
285 evolutionary analyses, the method is laborious and time consuming when investigating
286 multiple isolates. The development of new approaches to help streamline the MLST
287 workflow are very attractive, and recently we described how cross-referencing a refined four
288 gene MLST allelic profile to the full eight gene versions available in the MLST database at

289 <http://pubmlst.org/pacnes/> could be used to correctly predict and assign phylogroup, clonal
290 complex and, in the vast majority of cases, ST for a *P. acnes* isolate (38). In this context,
291 rapid pre-screening of isolates by multiplex PCR could also prove an extremely valuable way
292 to maximise the number of genetically diverse isolates selected for downstream MLST
293 analyses, thus reducing sequencing costs. Furthermore, MLST and whole genome analyses
294 have shown that types IA₂, IB, IC and III represent tight phylogenetic clusters, especially
295 when compared to types IA₁ and II (21, 22, 38) (Table S1); this is reflected in a more
296 restricted number of STs, some of which are highly dominant and widely disseminated. As a
297 consequence, high resolution MLST typing after multiplex PCR provides less phylogenetic
298 information for types IA₂, IB, IC and III isolates when compared to types IA₁ and II which
299 are genetically more heterogeneous and contain deeper levels of phylogenetic structure. In
300 keeping with the desirability of a simpler approach to high resolution typing of *P. acnes*, a
301 single locus typing scheme (SLST) for the bacterium based on nucleotide sequencing of an
302 amplified target region (484 bp) immediately upstream of the *campI* gene (identified by a
303 genome mining approach) was described during the preparation of this manuscript (55).
304 While the MLST₈ and MLST₉ schemes resolve a greater number of genotypes than SLST
305 (Table S1), the latter method does correctly cluster isolates into phylogenetic groupings that
306 are congruent with a core genome reference tree (55). In addition, there is little evidence of
307 recombination within the locus based on a network tree analysis (phi test p=0.976) (Fig S1).
308 The SLST method is, therefore, a valuable complement, and technically simpler approach, to
309 current MLST methods of typing *P. acnes*.

310 Rapid screening of isolates by multiplex PCR will also aid the discovery of novel taxa
311 via atypical PCR reactions. For example, sole reaction with the 16S rDNA primer set PArA-
312 1/ PArA-2 may indicate a new closely related species of *Propionibacterium* with high 16S
313 rDNA identity to *P. acnes*, or a novel *P. acnes* phylogroup or ST that contains base

314 mismatches at primer binding sites within the protein encoding genes of the assay. Indeed, as
315 a direct result of multiplex PCR screening of skin derived isolates, we have recently come
316 across such a scenario and identified a new *Propionibacterium* species that has very high 16S
317 rDNA identity to *P. acnes* (and reacts with PArA-1/ PArA-2) but is quite distinct from the
318 latter, and other cutaneous propionibacteria, based on whole genome analysis (currently
319 unpublished).

320 In conclusion, the multiplex PCR described here facilitates rapid molecular
321 confirmation of presumptive *P. acnes* isolates along with parallel phylogenetic typing. It
322 should provide researchers with a flexible typing tool that can be used in isolation, or as an
323 adjunct to more detailed sequence-based analysis depending on the epidemiological questions
324 being asked and resolution required. It is also a technically simple methodology for the rapid
325 analysis of mixed *P. acnes* populations, and should therefore help improve our understanding
326 of the role of different *P. acnes* lineages in clinical conditions.

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337

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FIGURE LEGEND.

525 FIG 1. Multiplex PCR analysis of *P. acnes* strains representing different phylogroups and
526 STs. Bottom lanes (left to right): 1, hdn-1 (ST1; type IA₁); 2, PRP-60 (ST20; type IA₁); 3,
527 76793 (ST101; type IA₁); 4, Pacn33 (ST2, type IA₂); 5, P.acn17 (ST2, type IA₂); 6, P. acn31
528 (ST2, type IA₂); 7, 6609 (ST5, type IB); 8, VA3/4 (ST78, type IB); 9, 74874 (ST43, type IB);
529 10, PRP-38 (ST70, type IC); 11, PV66 (ST85, type IC); 12, 5/1/3 (ST107, type IC); 13,
530 ATCC11828 (ST27, type II); 14, VA2/9N (ST28, type II); 15, 6187 (ST30, type II); 16, 12S
531 (ST32, type III); 17, Asn12 (ST33, type III); 18, Asn10 (ST81, type III); 19, *P. avidum*
532 44067; Ma molecular size markers. ST is based on the eight-gene MLST scheme of
533 McDowell et al. (21) and database at <http://pubmlst.org/pacnes/>. Gene amplicons (left to
534 right): a, 16S rRNA; b, ATPase; c, *sodA*; d, toxin; e, *atpD*; f, *recA*.

535 **TABLE 1. Multiplex PCR primer characteristics**

Primers ^a	Specificity	Gene targeted	Sequence (5'-3')	Position	Concentration	Annealing Temp (°C)	Amplicon size (bp)
PArA-1 PArA-2	<i>P. acnes</i>	16S rRNA	AAGCGTGAGTGACGGTAATGGGTA CCACCATAACGTGCTGGCAACAGT	442-465 1118-1095	0.2μM	66	677
PAMp-1 PAMp-2	IA ₁ /IA ₂ /IC	ATPase	GCGTTGACCAAGTCCGCCGA GCAAATTCGCACCGCGGAGC	451-470 944-925	0.25μM	66	494
PAMp-3 PAMp-4	IA ₂ /IB	<i>sodA</i>	CGGAACCATCAACAACTCGAA GAAGAACTCGTCAATCGCAGCA	168-189 312-291	0.6μM	62	145
PAMp-5 PAMp-6	IC	Toxin, Fic family	AGGGCGAGGTCCTCTTCTACCAGCG ACCCTCCAACGCAACTCTCCGCCT	17-41 321-297	0.1μM	66	305
PAMp-7 PAMp-8	II	<i>atpD</i>	TCCATCTGGCCGAATACCAGG TCTTAACGCCGATCCCTCCAT	339-360 689-669	0.15μM	66	351
PAMp-9 PAMp-10	III	<i>recA</i>	GCGCCCTCAAGTTCTACTCA CGGATTTGGTGATAATGCCA	641-660 865-846	0.25μM	66	225

536 ^aFor protein-encoding housekeeping genes, primers relate to positions within the open reading frame. For the 16S rRNA gene, primers relate to positions
537 within the sequence for NCTC737 (Genbank accession no. AB042288)

TABLE 2. Multiplex PCR assay accuracy

Phylogroup ^a	Number of isolates ^b		Sensitivity (%)	Specificity (%)
	Positive	Negative		
IA ₁	145/145	0/145	100	100
all others	0/216	216/216		
IA ₂	20/20	0/20	100	100
all others	0/341	341/341		
IB	65/65	0/65	100	100
all others	0/296	296/296		
IC	7/7	0/7	100	100
all others	0/354	354/354		
II	45/45	0/45	100	100
all others	0/316	316/316		
III	30/30	0/30	100	100
all others	0/331	331/331		

^aAll others relates to *P. acnes* isolates outside the target phylogroup, plus 49 isolates from other medically relevant species

^bPositive relates to detection of the expected amplification pattern under consideration, while negative indicates that one of the alternate phylogroup profiles was detected, or no reaction was observed in the case of other spp

